SIR1 and the Origin of Epigenetic States in *Saccharomyces cerevisiae*

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Silencing transcription of the majority of the genome in every cell is critical for normal growth, development, and regulation in multicellular eukaryotes. Current models of silencing build on the emerging details of chromatin modifications, as described in many of the papers in this volume. Modification states of histones, transcription factors, and other cellular proteins can strongly influence the recruitment and processivity of RNA polymerases.

Our goal in this short and decidedly noncomprehensive piece is to recap some insights from what has become a classic example of epigenetic control of transcription, that of silencing in the budding yeast *Saccharomyces cerevisiae*. Thorough and timely reviews may be found elsewhere (see, e.g., Rusche et al. 2003). Here, our historical and affectionate touchstone is the Silent Information Regulator gene, *SIR1*, which is pivotal in causing heterochromatin to assemble at and spread from silencers.

Most key players of silencing in yeast were initially identified mutationally. The subsequent molecular identification of some of their contributions to chromatin modification is mechanistically satisfying. Yet questions remain about other regulators, underscoring the probability of surprises in store as more is learned about how functionally different chromosomal states are established, maintained, and interconverted.

MUTANTS OF *SIR1* REVEAL EPIGENETIC SILENCING STATES

Historically, a popular approach to transcriptional studies was the biochemical identification of molecules and machines required for activation. These analyses provided much detail as in vitro reconstitution of transcription on purified DNA templates became increasingly refined. However, contemporaneously, genetic analyses provided examples in which the absence of transcription, rather than its activation, is essential and most relevant for normal cell function. One such key example in yeast is the existence of silent copies of the mating-type information. The purpose of these copies is not to be expressed. Rather, these genes serve as sources of genetic information that, when transposed elsewhere, determine cell identity.

The elegant “cassette hypothesis” developed by Ira Herskowitz and his colleagues explained many aspects of

the formal patterns through which cells could switch their identity by regulated access to the extra, although silent, copies of mating-type information (Herskowitz et al. 1977). Early versions of the hypothesis entertained the notion that something special about the mating-type locus, perhaps its promoter, caused expression at that locus. However, genetic studies soon turned the problem inside out with the discovery of recessive mutations that could activate the otherwise silent mating-type genes (Herskowitz et al. 1977; Haber and George 1979; Klar et al. 1979; Rine et al. 1979). What remained to be found, though, were the proteins necessary to prevent expression of the silent mating-type cassettes. Extended systematic analysis defined four major complementation groups of silent information regulators, the *SIR* genes, some alleles of which were also uncovered early on as *STE*, *MAR*, or *CMT* genes (Rine and Herskowitz 1987).

Even before molecular identification, the silent information regulator mutants fit into two categories: those with complete defects in silencing and one unusual case, *sir1*. In *sir1* mutants, silencing appeared to be only moderately affected in populations of mutant cells (Rine et al. 1979). Several different tests supported these distinctions between the mutants.

One way of assessing silencing is through mating tests in plate assays (Fig. 1). If haploid cells have normal silencing of the extra mating-type genes at *HML* and *HMR*, they mate with cells of the opposite mating type. If silencing is disrupted, haploids have many characteristics of diploid cells and fail to mate because they express mating-type information of both cell types. Under standard lab conditions, mating assays are performed so that the strains being tested have nonoverlapping auxotrophic markers. Therefore, mating is detected by selecting for prototrophic growth, which can occur only if a diploid has successfully formed by mating of the two haploids. In the example shown, *sir2Δ* mutants are completely mating defective, whereas, *sir1Δ* mutants appear to mate with an efficiency comparable to that of wild-type cells. However, this assay is blind to partial reductions in mating efficiency.

At first blush, a simple but incorrect explanation for the apparently incomplete effect on mating is that the *sir1* mutant alleles tested were only partial loss-of-function mutants, rather than null alleles that might completely destroy silencing. Therefore, when null alleles of *SIR1* were

Figure 1. Silencing in populations of cells is easily assayed in patch mating tests. Wild-type and *sir1Δ* mutant cells grow well, as shown in the control growth patches on rich medium in the *top* row. In mating tests for silencing, cells grow only if they successfully mate and form diploids with a wild-type cell of the mating type. In this example, as shown on the *bottom* row, *sir2Δ* mutants are completely mating defective in comparison to wild-type and *sir1Δ* cells. By other assays, *sir1* mutants were initially identified as silencing defective (Rine et al. 1979; Rine and Herskowitz 1987), although these defects are masked in population assays such as these. In quantitative mating assays or single-cell analyses (see text and Pillus and Rine 1989), 20–40% of *sir1Δ* cells are silencing competent, whereas the remainder of the population appears as defective as *sir2Δ* mutants.

found to have the same phenotype, all models based upon leaky alleles had to be discarded. Instead, two different views were equally compatible: (1) Sir1p played only a supporting role in silencing such that in all mutant cells the silent mating-type genes were activated to a slight extent or (2) in the absence of Sir1p, some cells silenced the *HML* and *HMR* loci and some did not.

In 1989, after many happy and, at times, perplexing hours and days in the company of single *sir1Δ* mutant cells at the micromanipulator, we concluded that *SIR1* provided insight into important aspects of transcriptional regulation. Notably, epigenetic transcription states could be a fundamental part of the biology of single eukaryotic cells.

In normal haploid cells, with functional silencing, in the presence of mating pheromone of the opposite cell type, the cells undergo cell-cycle arrest and characteristic morphological alterations in preparation for mating. This acutely sensitive assay for transcriptional status allows silencing to be measured in single mutant cells, their siblings, and their progeny simply by examining them microscopically.

In “Epigenetic inheritance of transcriptional states in *S. cerevisiae*,” we reported that seemingly identical cells from an isogenic population of *sir1Δ* mutants had two decidedly distinct transcriptional states: those that appeared to be completely normal and silenced, and those that were as defective in silencing as their *sir2*, *sir3*, or *sir4* mutant brethren (Pillus and Rine 1989).

Importantly, cells of either *sir1Δ* phenotypic class were mitotically stable with respect to their transcriptional silencing of mating-type information. This stability could last for many generations. Yet cells of either state, silenced or expressed, could also “switch” to assume the opposite phenotype at a low frequency, on the order of

once in every 250 cell divisions. Therefore, it appeared that rather than existing as some sort of continuum or threshold phenomenon, silencing was an either/or, on/off biological switch.

Furthermore, either transcriptional state could be propagated through meiosis. There was not any requisite, wholesale reprogramming of silenced states to move successfully through this major developmental process. Because either on or off transcriptional state could be sustained in *sir1* mutants, we concluded that Sir1p was not necessary for the maintenance of silencing, but rather was more likely to play a key role in its establishment. This point has caused some confusion over the years. After all, the two populations observed in *sir1Δ* cells could result from defects in either the establishment or the maintenance of the repressed state. In principle, one could imagine that establishment of silencing is ordinarily slow or inefficient in wild-type cells, but that maintenance is efficient, whereas maintenance is inefficient in *sir1Δ* cells.

The link of *SIR1* function to establishment is supported by two additional observations. First, in the small subset of cells in which the silenced state is established in *sir1Δ* cells, all cells in that generation are silenced rather than a small fraction. Second, under conditions in which *SIR1* function can be restored, such as with regulated promoter fusions, silencing is efficiently restored to all the *sir1Δ* cells in the population (Fox et al. 1997; Kirchmaier and Rine 2001).

ADDITIONAL EPIGENETICALLY REGULATED LOCI REVEAL COMMON FEATURES

As the molecular identification of *SIR1* was being finalized (Stone et al. 1991), independent observations were made that the epigenetic transcriptional states revealed by *sir1Δ* cells were not likely to be restricted to loss of *SIR1*. Indeed, regulatory site mutations at the silent mating-type loci, and expression of reporter genes at telomere-proximal sites revealed further fundamental possibilities for dynamic epigenetic changes in gene expression in yeast (Gottschling et al. 1990; Mahoney et al. 1991). The early studies of telomeric position effects paralleled those of *sir1* mutants and silent mating-type control. Indeed, both mitotic stability and distinct switches between states were observed in normal yeast cells. Telomeric silencing was also dependent on *SIR2*, *SIR3*, and *SIR4*, underscoring the potential coordinate function of these genes, although the epigenetic character of telomeric silencing is *SIR1* independent (Aparicio et al. 1991).

The possibility of an expanded role for *SIR* genes was also recognized in studies defining *SIR2* as a suppressor of recombination between the repetitive array of rDNA repeats within the nucleolus (Gottlieb and Esposito 1989). Although yeast cells are ordinarily characterized by high levels of homologous recombination in both meiotic and mitotic cells, suppression of recombination in the rDNA helps assure optimal numbers of the repeats and thereby coordinates regulation of ribosomal inventories, protein synthesis, and growth. A recombinational silenc-

ing role in the nucleolus is restricted to *SIR2*, since rDNA recombination is not affected in *sir1*, *sir3*, or *sir4* mutants. *SIR2* suppresses not just recombination, but also transposition, and transcription of pol II-driven reporter genes within the rDNA (Bryk et al. 1997; Fritze et al. 1997; Smith and Boeke 1997). Like recombination, these functions are intact in other *sir* mutant cells.

SIR2 is required for silencing at all three major genomic regions in yeast subject to epigenetic control, a role now known to be tied to its catalytic properties as an NAD⁺-dependent protein deacetylase of histones and other substrates. Significant progress has been made understanding the mechanisms of Sir2p's activities and the broader biological roles for *SIR2* and its relatives in yeast and in organisms ranging from bacteria to vertebrates (for recent reviews, see Blander and Guarente 2004; North and Verdin 2004).

THINKING ABOUT *SIR1* MECHANISTICALLY

Unlike *SIR2*, *SIR3*, or *SIR4*, which act in more than one region of the genome, from early studies, *SIR1* was distinguished by its apparent restriction to function at the silent mating-type loci. The *SIR1* gene can encode a 678 amino acid open reading frame (ORF), with no telltale structural domains or predicted catalytic activities (Stone et al. 1991). However, functional studies define an interaction between Sir1p and the DNA-binding Origin Recognition Complex (ORC; Triolo and Sternglanz 1996; Fox et al. 1997). The discovery of this relationship was particularly appealing because of the longstanding connection between the requirement for passage through S phase for the reestablishment of silencing after its disruption (Miller and Nasmyth 1984), the role of ORC in silencing (Foss et al. 1993), and the role of Sir1p in establishment. The requirement for both S phase and ORC for silencing suggested early notions of a requirement for DNA replication in silencing, but these proved incorrect (Kirchmaier and Rine 2001; Li et al. 2001). Models emerging from these observations and others (Chien et al. 1993) suggested that targeting Sir1p to silenced regions either through tethering to site-specific DNA-binding proteins or through the naturally targeted ORC complex is a critical feature of silent chromatin formation.

Indeed, both a genetic screen and molecular dissection of *SIR1* reveal a carboxy-terminal region of Sir1p that is critical for its interaction with Orc1p, the largest subunit of ORC, and with Sir4p (Gardner et al. 1999; Bose et al. 2004). These specific interactions, together with the low abundance of Sir1p (Gardner and Fox 2001), may help explain its restricted genomic functions. Furthermore, although Sir1p does not appear to be conserved at the level of primary amino acid sequence in multicellular eukaryotes, the regions identified as critical for interaction with ORC are conserved in other *Saccharomyces* species, as are two-hybrid interactions between the *Saccharomyces bayanus* and *Saccharomyces mikatae* Sir1 proteins and *S. cerevisiae* Orc1p. Tellingly, the most divergent ortholog is from *Saccharomyces castellii*, and in this case, where five amino acid substitutions are found in the critical interaction

region, the two-hybrid interaction is lost (Bose et al. 2004).

Roles beyond transcriptional silencing for Sir1p have been revealed by its occupancy in centromeric chromatin (Sharp et al. 2003). Interestingly, Sir1p appears to act independently of the other Sir proteins, because they are not found in centric chromatin. Furthermore, Sir1p binds to the Cac1p chromatin assembly factor and stabilizes its centromeric association. This binding may be functionally significant. Although chromosome stability is normal in single mutants, the *sir1Δ cac1Δ* double mutant has elevated nondisjunction rates that are further exacerbated when Hir1p, another Cac1p-associated factor, is also mutant.

Unlike larger eukaryotes, *Saccharomyces* has active genes very close to centromeres. Hence, Sir1p function at the centromeres does not reflect silencing in the sense that *HML* and *HMR* are silenced. However, recombination is suppressed near centromeres, and transcription through centromeres can destroy their function (Panzeri et al. 1984; Snyder et al. 1988). Thus perhaps Sir1p works with other as-yet-undefined proteins to repress recombination at centromeres, possibly in partnership with one of the *HST* cousins of *SIR2*. Alternatively, perhaps it nucleates a nonspreading chromatin structure that helps insulate centromeres from an occasional errant RNA polymerase transit.

ENHANCERS OF *sir1Δ* MUTANTS MAKE MORE CHROMATIN CONNECTIONS

Even in the absence of a detailed understanding of *SIR1*'s molecular mechanism, it seemed likely that the phenotype of the null mutants could give more insight into the epigenetic control of silencing. For example, what protein or process fulfills Sir1p's role in establishment in the fraction of cells that silence *HML* and *HMR* in *sir1Δ* mutants?

One approach to answering this question was to identify additional mutants that themselves were mating competent, but in combination with the *sir1Δ* mutation would become completely mating defective (Reifsnnyder et al. 1996). In this screen for enhancers of the *sir1Δ* phenotype, nonnull alleles of *SIR2*, *SIR3*, and *SIR4* were identified, some of which have been studied in detail and give insight into locus-specific requirements for these genes (Stone et al. 2000; Garcia and Pillus 2002). Significantly, an allele of the *SAS2* gene was recovered. Other alleles of *SAS2* had been identified independently as suppressors that restore mating to strains bearing a defective, sensitized *HMR cis*-silencer sequence (Ehrenhofer-Murray et al. 1997). Both studies found that *sas2* mutants completely eliminate silencing of telomeric reporter genes. The fact that Sas2p functions may be positive in the case of *sir1Δ* mutants and at telomeres, but antagonistic at *HMR*, underscores that it has distinct roles depending on the region of the genome where it acts.

Sas2p is part of the MYST family, so named for its founding members, the leukemia-associated *Moz* protein, the Yeast *Sas* proteins, and Tip-60, a human protein first found as an interactor with the HIV Tat transactivator.

The MYST family is broadly conserved, with two closely related proteins in yeast, Esa1p and Sas3p, and multiple orthologs in every eukaryote for which sequence information is available. Relatively weak sequence similarity to proteins with acetyltransferase activity led to the discovery of histone and nucleosome acetyltransferase activity for all three yeast paralogs and many multicellular orthologs. These orthologs contribute to such diverse processes as dosage compensation, apoptosis, and response to DNA damage (for review, see Carrozza et al. 2003; Utley and Côté 2003).

MYST family proteins, including Sas2p, are found in complexes whose subunits contribute to activity and specificity. In the case of Sas2p's partners, Sas4p and Sas5p, the mating defect of *sir1Δ* cells is enhanced by mutants of either subunit (Xu et al. 1999a,b).

Other enhancers of *sir1Δ* also have connections to chromatin modification. *NAT1* and *ARD1* are required for mating in *sir1* mutant backgrounds (Whiteway et al. 1987; Stone et al. 1991). Nat1p and Ard1p are components of an amino-terminal protein acetyltransferase (NatA) with many cellular substrates (for review, see Polevoda and Sherman 2003), including a recently described role acetylating Sir3p and Orc1p (Wang et al. 2004). Further, the *sir1Δ cac1Δ hir1Δ* mutant introduced above not only affects centromeres, but is also completely mating defective (Kaufman et al. 1998). The loss of mating in these additional mutant backgrounds underscores the idea that Sir1p activity becomes critical for silencing in many genetically compromised circumstances, including in cells with synthetic versions of silencers (McNally and Rine 1991).

We discovered a circumstance requiring *SIR1* that points to a previously unsuspected role of higher-order structure or topological constraints in silencing. Both of the silent mating-type loci, *HML* and *HMR*, and the actively transcribed *MAT* locus reside on chromosome III. Homologous recombination between *HML* and *HMR* results in circular derivatives of the chromosome (Strathern et al. 1979; Newlon et al. 1991) in which only genetic information between the silent loci and telomeres is lost. Fortunately, there are no essential genes distal to these loci. Thus, strains carrying a large circular chromosome III are fully viable and mating competent, yet have no free chromosome ends or telomeres on this chromosome. We observed that in such strains, mutation of *SIR1* results in loss of mating (Fig. 2). Thus, changing chromosomal structure renders silencing completely dependent on *SIR1*. The mechanism of this dramatic effect is not yet clear, but may result from topological changes, altered localization, altered replication timing, or other higher-order influences on chromosome structure.

ANALOGS BUT NOT HOMOLOGS OF *SIR1*

Given that heterochromatin is common to all eukaryotes, and that *SIR2* family members seem to participate in heterochromatin in many if not all of these, it is striking that *SIR1* homologs have not been found outside of the *Saccharomyces* genera. If it is so central to the establish-

Figure 2. *SIR1* is required for mating-type silencing when chromosome III is circular. Unlike the normal genomic context of a linear chromosome III (as in Fig. 1), *sir1Δ* mutants become completely mating-defective when chromosome III is circularized by recombination between homologous sequences of the silent mating-type loci. Cartoons of chromosome III (top) highlight the telomeres (T), the centromere (C), and the silent mating-type loci *HML* and *HMR*, on the left and right arms of the chromosome. Transcription of the centromere-proximal *MAT* locus defines cell type. In the circular chromosome, recombination has occurred between the sites highlighted by lightning bolts. Although wild-type and *sir1Δ* mutants are healthy with this circular derivative (top), the *sir1Δ* mutants fail to mate (bottom).

ment of heterochromatin in *Saccharomyces*, what plays this role in other species?

A reasonable argument can be made for HP1 being a Sir1p analog. HP1, so named as the first discovered heterochromatin protein, found initially in *Drosophila*, is required for heterochromatin function in both flies and in *Schizosaccharomyces pombe* (for review, see Eissenberg and Elgin 2000; Kellum 2003; Maison and Almouzni 2004). In *Drosophila*, HP1 binds to the amino terminus of the Orc1 subunit of ORC analogously to the way that Sir1p binds to ORC, and *orc* mutants in *Drosophila* function disrupt heterochromatin (Shareef et al. 2001; Prasanth et al. 2004). Moreover, the heterochromatin function of ORC subunits is functionally interchangeable between yeast and *Drosophila* (Ehrenhofer-Murray et al. 1995; Pak et al. 1997). Hence, there seems to be a deep and conserved connection between proteins that can initiate heterochromatin and ORC. It is curious that a link between *S. pombe*'s ORC and heterochromatin has not been made, especially given Sir2p's requirement for heterochromatin formation in *S. pombe* (Shankaranarayana et al. 2003; Freeman-Cook et al. 2005).

From a different perspective, perhaps it is more surprising that *Saccharomyces* does use Sir1 and its paralogs to enhance the efficiency of silencing. After all, a mechanism of repression that has no way for being turned on and off would not be useful for regulating genes that need to be turned on under some conditions but not others. This consideration leads us to suggest that Sir protein-based silencing may have evolved without Sir1 to allow for the

variable yet heritable expression states of certain genes such as those turned on under some conditions but not others, including subtelomeric genes, such as the *FLO* genes of *Saccharomyces* and adhesion genes of *Candida* (B. Cormack, pers. comm.). Sir1 would then be a protein whose function is to push the equilibrium between the assembly of silenced chromatin versus active chromatin decidedly in the direction of silencing for genes whose purpose is not to be expressed, but to serve as a donor in the repair of double-stranded breaks at *MAT*. It will be interesting to see if HP1 serves a similar linchpin role in recruiting intercalary heterochromatin proteins to sites of ORC localization in *S. pombe* and *Drosophila*.

MORE *SIR1*s, MORE EPIGENETICS

The accumulating evidence from *sir1Δ* mutants and their enhancers demonstrates that loss of *SIR1* sensitizes transcriptional silencing to require other structural and catalytic regulators of chromatin for efficient silencing. Elements of Sir1p's functions at the *HML* and *HMR* silencer elements are clearly related to its recruitment to those sites by Orc1p and Sir4p. Precisely what does Sir1p do when bound? Most current models propose kinetic roles in the assembly of silent chromatin. For example, by providing an extra kilocalorie or so of free energy, Sir1p's interactions with ORC and Sir4p may overcome a kinetic barrier to the formation of silenced chromatin.

Recent studies cited above suggest that Sir1p has a broader spectrum of function than previously suspected. Sir1p's contribution to centromere function is independent of the other Sir proteins (Sharp et al. 2003). Enhancer screens in *sir1Δ* mutants focused on chromosome loss or segregation phenotypes may yield deeper insight into epigenetic aspects of centromere functions in yeast.

It has not yet been established whether the same regions of Sir1p defined for silencer binding are required for its centromeric functions or for functions with the circular derivative of chromosome III. Any differences might help define specificity of targeting and functionally distinct genomic interactions. Indeed, the *SIR1* gene may encode more than one species of protein. Specifically, *SIR1* produces two transcripts, the shorter of which is twice as abundant as the longer (Ivy et al. 1986; Stone et al. 1991). The more abundant transcript, if it is translated, would yield an alternative Sir1p isoform with a disrupted Orc1p-Sir4p binding domain (Sparks and Dieckmann 1998). Multiple in-frame methionine codons near the 5' end of the *SIR1* ORF amplify the diversity of potential proteins from the locus. Determining whether other Sir1p isoforms have distinct or interfering functions in silencing may provide new insights into previously defined epigenetic states or those that control other less well understood biological processes.

Indeed, epigenetic control of pseudohyphal switching was recently reported to be influenced by the Hst1p and Hst2p members of the Sir2p family (Halme et al. 2004). Genes regulated by these deacetylases are relatively telomere proximal, regions known from several genome-wide studies to be particularly sensitive to histone acety-

lation state (see, e.g., the review by Millar et al., this volume). It seems reasonable to expect that as understanding of physiological states becomes more fully integrated with knowledge of transcriptional regulation, additional roles will be uncovered for molecules like Sir1p that may have the capacity to buffer epigenetic states. Moreover, the *Saccharomyces* genus offers unusually favorable opportunities to uncover new roles for Sir1p-like functions as up to four paralogs of *SIR1* can be found within its species.

Although the epigenetic aspects of silencing revealed by *sir1* mutations have been the focus of this discussion, the underlying on-or-off nature of silencing is also of interest as it bears on whether repression mechanisms have analog or digital qualities. This issue has received some attention. For example, the *GAL1* gene of *S. cerevisiae* is repressed in medium containing glucose by two different repressors, Gal80p and Mig1p. In the absence of Gal80p, glucose repression of *GAL1* is graded, whereas in the absence of Mig1p, it is on or off (Biggar and Crabtree 2001). Hence the quality of gene repression in response to a physiological stimulus can be either analog or digital, depending upon which repressor mediates the signal. The studies of *SIR1* suggest that mutants with "leaky" phenotypes can be particularly informative regarding mechanisms of repression, but capitalizing on such opportunities requires assays that can be performed robustly at the single-cell level. The versatility of mating-type regulation makes it supremely amenable to such studies.

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